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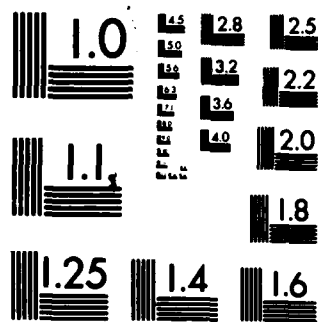
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MORPHOGENESIS OF DENGUE VIRUS:
Molecular Biology and Molecular Organization of Proteins

Final Report

February 1981

(For the period November 1971 to July 1980)

Principal Investigator

JaRue S. Manning, Ph.D.

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Our findings then led us to postulate that DEN antigens on the cell surface were composed of ordered arrays of DEN polypeptides. As a first approach we proposed to examine the surface of the virus itself and then proceed to a study of the cell surface.

To do this we studied the growth of DEN in BHK cells. Our findings established new growth conditions and assay procedures for the virus. We report this here in Section III.

Since our approach was to use crosslinking reagents to study the organization of the DEN polypeptides we examined the charge properties of viral glycoproteins on two other togaviruses. This was done since it was believed that the charge distribution could influence organization of viral polypeptides and their crosslinking. Results of these studies showed that the glycoproteins of two closely related viruses were quite different but that these differences probably would not significantly alter the organization of polypeptides on the surface of the virion. These findings are reported in Section IV.

Finally, we applied the knowledge obtained in the above studies to the analysis of protein-protein interactions on Japanese encephalitis virus (JEV), a virus closely related to DEN. These results demonstrated that the major viral glycoprotein on the surface of the virion had as its nearest neighbor either one of its kind or another newly defined viral glycoprotein. Thus the clustering of viral antigens on flaviviruses had been demonstrated. This is reported here in Section V.

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I. Introduction and Summary

Research supported by this Contract resulted in 4 major advances in our understanding of Togaviruses among which is DENGUE virus (DEN). Each of these major advances is described in some detail and each has or will be included in a published manuscript which acknowledges the source of support.

The first major finding was the appearance of DEN antigens on the surface of infected cells. This is reported in Section I. This was of major importance since it has been hypothesized that dengue hemorrhagic fever (DHF) is the result of DEN antigen interaction with anti DEN antibody resulting complement mediated vascular endothelial injury.

Our findings then led us to postulate that DEN antigens on the cell surface were composed of ordered arrays of DEN polypeptides. As a first approach we proposed to examine the surface of the virus itself and then proceed to a study of the cell surface.

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II. Surface Labeling of Dengue Virus Infected Cells

The flaviviruses are important human pathogens. An understanding of the intracellular distribution of Flavivirus antigens has been critical in the interpretation of certain disease states, particularly dengue virus infections and immune mediated dengue disease syndromes (1).

Like other flavivirus, dengue virus matures within the cytoplasm of host cells in association with the endoplasmic reticulum and the Golgi complex. Release of virus appears to be by reverse pinocytoses (2, 3). Maturation by budding through internal or external cell membranes has not been observed. Fluorescent antibody studies have revealed intense staining at the perinuclear region of the cytoplasm with little or no visible plasma

membrane staining (2). These observations implied that infected cells did not carry dengue antigens on the cell membrane (3).

Recent evidence has indicated, however, that virus-related antigens do exist on the surface of dengue infected cells. Stohlman, et al. found radiolabeled dengue proteins in plasma membrane fractions of dengue virus infected BHK cells (4). Catanzaro, et al. demonstrated dengue specific plasma membrane antigens using immunoperoxidase and immune cytotoxicity techniques on viable LLC MK2 cells (3). Brandt and Russell have confirmed and extended these observations to Japanese encephalitis virus using immune cytotoxicity (5). These last two studies have been confined to cell surface antigens and have utilized antibodies against crude extracts of dengue-2 infected mouse brain (anti-DEN-2).

In addition to anti-DEN-2, other hyperimmune ascitic fluids have been produced against purified virions (anti-RHA) and against a non-structural dengue (anti-SCF) antigen. All three antibody systems have been previously characterized (2, 6).

The most significant aspect of this study was the identification of both virion (RHA) and non-virion (SCF) antigens, on the surface of dengue-2 infected LLC MK2 cells. These studies corroborate and extend similar biochemical observations with immunological data (4). The non-virion SCF is of particular interest since it has been regarded as an antigen which might be associated with dengue hemorrhagic fever or shock syndrome (1, 8, 9). The presence of SCF on the surface of dengue infected cells increases the likelihood of host response to this antigen. Such an immunological response against SCF has been observed in patients with secondary dengue virus infections (7). The host immune response could be directly against the cell surface RHA or SCF antigens creating an in-vivo immunocytotoxicity (3, 5). Alternatively, an external position could facilitate antigen release (9). Circulating antigens could combine with antibodies to make immune complexes which have been discussed in the pathogenesis of dengue shock syndrome (1).

The diffuse nature of the membrane RP appears to conflict with the previous observations of focal RP (6). It should be emphasized that the present study utilized prefixed cells, reacted in-situ, while the study of Catanzaro, et al. (3) used viable cells which were fixed after the immunological reactions were completed. The distribution of the membrane RP could well reflect the relative mobility of the antigens in the membranes of fixed and unfixed cells (9).

The interpretation of the membrane reaction must be tempered by the recognition that all three primary HMAF had antibodies against mouse brain antigens. The most rigorous means of circumventing this problem would have been extensive adsorption of the HMAF with mouse brain. The limited amount of HMAF available and the many negative controls encouraged us to work with the HMAF at high dilutions. Most of the zero hour postinfection cells and most of the mock-infected cells were negative. As has been illustrated, however, some of the control cells did have a slightly positive membrane reaction. While the majority of negative control cells and the very light RP in the positive control cells provide evidence of specificity, a

contribution from residual mouse brain antigens cannot be excluded in any given positive cell in the dengue infected cultures.

An intracytoplasmic IP reaction was also found using all three HMAF. The pattern of the IP RP was clearly dependent upon the extent of penetration of the immunoglobulins into the cytoplasm. The three dimensional reconstructions demonstrated that the cytoplasmic RP could be found only in cells with disrupted membranes. Penetration must be considered the primary cause of polar RP and negative virions, cytopathic vacuoles or other non-reactive areas. As a result, areas or organelles without IP RP must be regarded as inadequately penetrated areas rather than antigen negative areas.

The areas of the cell which were adequately penetrated with immunoglobulins revealed a diffuse granular cytoplasmic RP. This pattern of RP could be attributed either to antigens soluble in the cytoplasm and/or to membrane free polyribisomes as sites of protein synthesis. In either case, the dengue-2 antigens were not limited to the membranes of the endoplasmic reticulum as is implied by biochemical studies of dengue and of other flaviviruses (4, 10).

These studies established that both virion and non-virion antigens appear on the plasma membrane of dengue infected cells. They correlate well with the findings of radiolabeled dengue proteins into plasma membranes (3). The hypothesis that these are membrane-bound antigens which are inserted into the surface membranes during virus expulsion by reverse pinocytoses (exocytosis) (8), would predict that the appearance of the surface antigens would correlate with virus release. The appearance of surface antigens as measured by immune cytolysis does not, however, necessarily correspond with the release of infectious virus (3, 9). These discrepancies might be resolved if not all antigens were bound to the endoplasmic reticulum. The presence of non-membrane, cytoplasmic, dengue specific antigens as described above could lead to direct antigen insertion into the plasma membrane quite independent of virus release by exocytosis. While these experiments provide conceptual alternatives, much more work is required to clarify the molecular biology of Flaviviruses.

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III. Properties of DEN-2 Grown in BHK Cells

Dengue virus (DEN), a arthropod-borne flavivirus, is an agent recognized as responsible for benign dengue fever as well as life-threatening dengue hemorrhagic fever and dengue shock syndrome. Four serotypes of DEN have been identified and all four serotypes have been recovered from patients with mild and severe dengue illnesses (P. K. Russell, personal communication). A variety of cells that can be propagated in cell culture support the replication of DEN (1,2,4,5,8,17-19) and several clinical techniques for the isolation and serological identification of the virus are based upon cell culture methods (3,4,6,11,18).

Although conditions for the plaque assay of DEN have been detailed (12-16), quantitative information is limited on the stability of the virus to common cell culture and laboratory environments. The present paper describes the adaptation of the virus to growth in BHK cells and use of a tragacanth gum plaque assay. Results of studies on the stability of type 2 DEN when held at temperatures of 4° and 37°, pH 6.5 through 8.0, diluted in buffers varying in divalent cation concentration, and when subjected to multiple freeze-thaw cycles are presented and discussed.

Plaque assay. DEN-2 was titrated by plaque assay on BHK-21/15 cells using a semisolid tragacanth gum overlay. A 5 day incubation period was found to be optimal for obtaining readily detectable plaques. Non-specific degeneration of the monolayer was not encountered under the assay conditions used.

During the development of the gum plaque assay procedure was observed that cell cultures inoculated with virus when subconfluent resulted in substantially larger plaques. Cultures which were approximately 80% confluent at inoculation yielded plaques ranging in size from 0.5 mm to 3.5 mm, while cultures confluent at inoculation produced plaques ranging from 0.5 to 1.5 mm in diameter. The heterogeneity in DEN-2 plaque size was consistently observed. Cultures subconfluent at inoculation grew to confluency during the 5 day assay period, as is evident from the staining

pattern. No difference in plaque titer was obtained when confluent and subconfluent cultures were compared.

Serial Passage in BHK-21/15 cells. Virus obtained as seed stock in SMB suspension was adapted to BHK-21/15 cells by serial passage. Seed stock DEN-2 was inoculated onto cell monolayers. Tissue culture fluid (TCF) was removed 72 hours post-infection when CPE was apparent. The TCF was cleared of cells by centrifugation at 2000Xg for 10 min. An aliquot of the cleared TCF was inoculated onto a new cell monolayer. The TCF was harvested and prepared for subsequent passage as above. Six serial passages of DEN-2 in BHK-21/15 cells were made. Virus titer of the successive serial passages was then determined.

Table 1 lists the titers obtained at different passage numbers as well as the moi. To minimize production of defective virions, serial passage was performed using low moi. The percentage of cells showing CPE at 72 hr postinfection increased substantially during serial passage. Approximately 30% of cells demonstrated CPE during the first and second passages. By the sixth passage the entire cell monolayer was affected.

Multiplication cycle of BHK cell-adapted virus. The growth curve of DEN-2 propagated in BHK-21/15 cells was determined. BHK cells were inoculated with virus (passage 6) at an loi of 10. Small (0.1 ml) aliquots of TCF (20 ml initial volume) was harvested at 12 hr intervals post-infection. The TCF was prepared and assayed for virus as described above. Extracellular DEN-2 was detected in the medium at 24 hr post-infection. Maximum titers were found between 36 and 60 hr. After 60 hr the titer of infections extracellular virus rapidly decreased.

Stability of infectivity at 4° and 37°. Since dengue virus is grown in vertebrate cells in cell culture at or near 37° and the virus often stored at 4°, we examined the effect of these temperatures on survival of infectivity. Aliquots of TCF from virus infected cells which had been cleared by low speed centrifugation were placed into small vials and sealed. The vials were held at either 4° or 37° for different intervals. The vials were then immediately frozen at -70° prior to plaque assay.

Virus in TCF maintained at 4° for 24 hr showed no reduction in titer. A reduction of about 40% was detected for virus held at 4° for 48 hr. In contrast, DEN-2 held at 37° was rapidly inactivated. Following incubation at 37° for 12 hr, only 30% of the initial infectivity remained. Less than 0.01% of the infectivity survived 24 h at 37°.

Influence of pH. During virus growth in culture cell metabolism often leads to a change in the pH of the growth medium. To determine the effect of pH on DEN-2 infectivity, virus infected TCF cleared of cells was diluted 100 fold with MEM containing 10% FCS buffered to pH 6.5, 7.0, 7.5, or 8.0. Vials containing the diluted virus were sealed and incubated at 4° for 24 hr. Prior to plaque assay all samples were adjusted to pH 8.0. Table 2 illustrates a small but progressive decline in infectivity with reduction in pH. Loss of infectivity during incubation at 4° for 24 hr at pH 6.5 amounted to 70% of the titer of virus maintained at pH 8.0.

Influence of divalent cations. During virus isolation and characterization chelating agents which alter divalent cation concentration are occasionally used. To determine the effect of divalent cations on dengue infectivity, TCF containing DEN-2 was cleared of cells and diluted 1000 fold in the following solutions: Tris buffered saline (TBS), pH 8.0; TBS containing 1mM $MgCl_2$ and 1 mM $CaCl_2$; TBS containing 1mM EDTA; MEM containing 10% FCS. The diluted samples were placed in vials, sealed and then held at 4° for 24 hr. All samples were diluted in serum-free MEM pH 8.0 before plaque assay.

As shown in Table 3, DEN-2 infectivity was not substantially changed when diluted in TBS or TBS with $MgCl_2$ and $CaCl_2$. However, infectivity was reduced by nearly 3 \log_{10} PFU/ml when the TBS diluent was supplemented with 1 mM EDTA.

Effect of multiple freeze-thaw. Dengue virus isolates are both routinely transported and stored at temperatures of -70° to -80° and are occasionally subject to several cycles of freezing and thawing. The effect of multiple freeze-thaw cycles on DEN-2 infectivity was examined by assaying virus for infectivity in TCF after several cycles of freezing at -75° and thawing to 25°. An ampoule containing a thermocouple immersed in TCF was used to monitor temperatures. DEN-2 infectivity was stable under the test conditions. No reduction in titer was noted after 1, 2, or 3 cycles of freezing and thawing.

The data presented above describes the adaptation of DEN-2 to growth in BHK-21/15 cells and the stability of infectivity to cell culture and laboratory conditions. Viral infectivity was monitored by plaque assay under a semisolid tragacanth gum overlay. In developing the plaque assay it was noted that plaque size at the end of the 5 day incubation period was related to the degree of monolayer confluency at time of virus inoculation. Although DEN plaque assays yield a range of plaque sizes (13), the size range was appreciable greater for cultures subconfluent at inoculation as compared with those which were confluent. In a previous study (15) monkey kidney cell cultures inoculated on day 3 or 24 after transfer resulted in little difference in plaque titer. Any variation in plaque size that might have occurred was not reported; however, given the cell transfer conditions used, it is likely that cultures were confluent on both day 3 and 24. We suggest that subconfluent cultures may be helpful in enumerating virus infection where very small diameter plaques are encountered when standard confluent monolayers are used and would also shorten the incubation period for plaque assay of these viruses.

Often high titers of virions grown in cell culture can only be obtained after adaptation by serial passage. BHK cells are susceptible to DEN from suckling mouse brain suspensions and have been used in plaque assays (6,9) and morphological studies (7). On the basis of this information we serially passaged DEN-2 in BHK-21/15 cells to determine if the virus could be adapted to grow to high titers in such cells. The production of such high titered suspensions of virus in cell culture fluid would eliminate the conventional dependence on suckling mouse brain preparations as a source of virus. Our results demonstrated that high titer DEN-2 virus can be generated by sequential passage of the virus in BHK-21/15 cells with a titer of 10^7 PFU/ml of extracellular virus being present by passage 6.

The growth curve of DEN-2 in BHK-21/15 cells showed release of the virus by 24 hr with peak titers between 36 and 60 hr post-infection. The dramatic decrease in titer found in the present study between 60 and 84 hr post-infection is most likely due to thermal inactivation of virus already released into the medium. The temperature sensitivity study demonstrated a reduction of greater than $3 \log_{10}$ PFU/ml in 24 hr at 37° which would account for the decline assuming that virus replication and release has essentially been completed by 60hr.

The infectivity of DEN-2 was found to be dependent upon the pH of the suspension medium when maintained at 4° for 24 hr. The titer decreased as the pH was reduced (Table 2). This finding points up the importance in maintaining a basic pH when storing the virus. These studies were conducted at 4° to allow distinction between pH and temperature effects, but the results are most likely valid for virus replicating in cell culture at 37° . It has been previously reported that a variation in pH between 6.6 and 8.6 of the agar overlay in DEN plaque assay had little effect on plaque titer (15). The apparent discrepancy can be explained by experimental differences. In plaque formation it is unlikely that virus released from infected cells resides in the matrix medium for periods sufficient to show the pH effects as reported here.

The maintenance of DEN-2 infectivity in solution requires divalent cations as shown by the dramatic drop in virus titer following incubation in a TBS containing 1MM EDTA (Table 3). Since there was little change in infectivity following suspension in TBS, the divalent cations are most likely bound to the virion in a relatively stable manner. The ability of EDTA to reduce infectivity suggests that those essential divalent cations are located in the viral envelope and near the virion surface. The divalent cations probably act to stabilize viral envelope proteins, as recently found for feline leukemia virus (Durbin R. K. and J. S. Manning, unpublished data). The data presented suggests that chelating agents should not be used in the isolation and characterization of DEN.

The addition of Mg^{++} and Ca^{++} to TBS in which DEN-2 was suspended neither enhanced nor reduced virus infectivity. In two previous studies on DEN plaque formation it was reported that variation in Mg^{++} and Ca^{++} concentrations was without effect (16) and with slight enhancing effect (15), although the latter may have perhaps been due to effects of interfering components in the assay. More recently, the addition of divalent cation to the growth medium of DEN-2 infected Vero cells was reported to result in increased cell-free titers (9). The report suggests that the higher titers recovered from these cultures was due to an enhancement of virus maturation and release. In addition, the enhancing effect was restricted to virus infected Vero cells and was not observed when infected KB or BHK cells were tested.

Finally, it is known that DEN can survive for years when maintained at -70° to -80° temperatures (2). When DEN-2 in TCF containing 10% FCS was subjected to 3 cycles of freezing and thawing there was no detectable change in titer. Since DEN field samples are routinely stored in serum at -70° or lower, the data presented here indicates that DEN can be subjected to several cycles of freezing and thawing without deleterious effects.

TABLE 1

Titers of DEN-2 with Sequential Passage in BKH-21/15 Cells

Passage Number	Titer (PFU/ml)	MOI
1	6×10^{-3}	---
3	1×10^{-4}	0.002
5	1×10^5	0.008
7	3×10^7	0.02

TABLE 2

Effect of pH on the infectivity of Dengue Virus

pH ^a	PFU/ml
6.5	3×10^3
7.0	6×10^3
7.5	8×10^3
8.0	1×10^4

^a Virus was stored in complete medium with 10% FCS at the indicated pH for 24 h at 4°C.

TABLE 3

Effect of Divalent Cation Removal on the Infectivity of Dengue Virus

Treatment ^a	PFU/ml
None ^b	3×10^4
Complete medium	2×10^4
TBS + Ca^{++} + Mg^{++}	2×10^4
TBS alone	1×10^4
TBS + 1 mM EDTA	30

^a Virus in MEM was diluted one thousand fold in the indicated buffer and solutions and stored at 4°C for 24 hr. For titration all virus samples were diluted into complete MEM + 10% FCS before infection.

^b Untreated virus in TCF used in these studies was diluted 1000 fold in MEM with 10% FCS and assayed as per test samples.

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IV. Glycoproteins of Sindbis and Semliki Forest Virus

Sindbis virus (SV) and Semliki Forest Virus (SFV) are arthropod-borne alphaviruses of the togavirus family. Both viruses contain a nucleocapsid comprised of a core protein of thirty thousand daltons₆ (30 kd) molecular weight (6,14,15) and a single strand of RNA of 4.2×10^6 daltons (3,4,13). The envelope of SV contains two glycoproteins, E₁ and E₂, having molecular weight of 53 kd and 50 kd respectively (11,16). The SFV envelope contains three glycoproteins, E₁, E₂, and E₃, having molecular weights of 49 kd, 53 kd and 10 kd respectively (5). Carbohydrate contributes from 7 to 11 percent of the apparent molecular weight of E₁ and E₂ for both viruses and approximately forty-five percent of that of the SFV E₃ glycoprotein (5,12). Carbohydrate side chains are attached to the polypeptide chain by an N-glycosidic linkage to asparagine in SV; no O-glycosidic linkages have been found (12). Both galactose-rich type A and mannose-rich type B oligosaccharide side chains are present on the E₁ and E₂ glycoproteins of SV (12). Glycoproteins of both viruses contain sialic acid (6,12) terminally located on the carbohydrate side chains and bearing a negative charge at physiologic pH, however, distribution of sialic acid residues on the glycoprotein was unknown.

SV glycoproteins, E₁, and E₂, separated by density gradient-stabilized column isoelectric focusing have isoelectric points of pI 6 and pI 9 (2). Intact SV has an isoelectric point of pI 4.2 (2) while that of intact SFV has a pI of 6.2 (8). The isoelectric points for SFV glycoproteins have not been reported.

Preliminary isoelectric focusing studies in our laboratory using polyacrylamide gels revealed that both SV glycoprotein preparations were comprised of five charge isomers. We suspected that this apparent discrete charge heterogeneity might be due to differences in the degree of sialylation of glycoproteins. In this study we utilized isoelectric focusing combined with second dimension electrophoresis in polyacrylamide gels to examine the charge properties of SV and SFV glycoproteins. Protein concentrations were determined using standard methods (9).

Isoelectric focusing of Sindbis and Semliki Forest Virus polypeptides. Purified unlabeled SV and ³⁵S-methionine labeled SFV (10) were disrupted and

subjected to isoelectric focusing in polyacrylamide gels. Ampholytes of pH range 3.5-10 were used to form the pH gradient for the focusing of SV polypeptides. Viral polypeptides were resolved into three groups. Two of these groups were comprised of at least five distinct bands. The pH gradient obtained extended from pH 4.4 to 9.2. One group banding in the pH range 6.1 to 6.8, was considered to be E₁. The second group of polypeptides which focused in the range of pH 8.0 to 8.6 was believed to be E₂. Polypeptides focusing in the pH 8.0 to 8.6 range did not form bands as sharp as the polypeptides focusing in the pH 6.1-6.8 range. The protein with a pI value greater than 9.0 was considered to be the SV core protein.

When ³⁵S-methionine labeled SFV was focused in gels under the conditions used for SV three or occasionally four peaks were detected. These peaks focused as a single group in the pH range 6.7 to 7.5 and clearly indicating that the polypeptides of SFV had pI values distinct from those of SV. In order to enhance resolution of SFV polypeptides IEF was performed using a shallower pH gradient, generated by using a mixture of ampholytes with pH ranges of 7 to 9 and 3.6 to 10 in a ratio of 5.25 to 1.0. The resulting pH gradient was linear between pH 6.5 and 8.8. Under these conditions, SFV polypeptides separated into five major peaks with a distinct shoulder on the acidic edge of the peak at pH 7.0. The peak appearing at pH 5.4 was not consistently observed and may represent a complex of core protein and viral RNA. The shoulder (pI 6.9) and four peaks having pI values of 7.0, 7.2, 7.4, and 7.6 were believed to be charge isomers of the envelope polypeptides E₁ and E₂. Although the tentative assignments of E₁ and E₂ to the two groups of SV polypeptides separated in IEF gels was most likely correct in light of report by Dalrymple et al. (2), identification of the focused SFV polypeptides was not possible without further analysis. In order to unambiguously identify the polypeptides separated by isoelectric focusing, we utilized a second dimension slab gel system.

Acid-urea gel electrophoresis of SV and SFV polypeptides. An acid-urea polyacrylamide gel system was employed to identify the polypeptides separated in the IEF since this a highly reproducible method of resolving envelope proteins of both SV and SFV and it was not necessary to remove the ampholytes from pH 3 to 10 gels prior to electrophoresis in the second dimension. However, the nomenclature for the identification of SV and SFV polypeptides was established using SDS polyacrylamide gels (5,6,11), and it was therefore necessary to relate the order of electrophoretic migration of polypeptides in acid-urea gels to that in SDS gels. Unlike SDS gels, the mobility is related to both the size and charge on the polypeptide at the pH of the gel. SV and SFV polypeptides were well resolved in acid-urea gels. SV and SFV polypeptides were found to have distinctly different mobilities under these electrophoresis conditions. Correlation between the bands, and E₁, E₂, and core proteins was obtained by electrophoresis into a second dimension SDS gel. Lanes cut from unfixed acid-urea slab gels containing the separated viral polypeptides were equilibrated with disruption buffer as described above and placed onto SDS slab gels such that electrophoresis in the SDS gel would be in a direction perpendicular to the direction of migration in the acid-urea gel. The SV polypeptides were found to migrate in the same order in both acid-urea and SDS gel systems. However, second dimension analysis of SFV polypeptides revealed that E₁ and E₂ were reversed in the order of migration. It should be noted that the nomenclature for SV and SFV envelope polypeptides is different (5,11). In

SDS gel SV E₁ migrates most slowly whereas E₂ is the most slowly migrating polypeptide of SFV.

Identification of the isoelectrically focused polypeptides. Viral polypeptides which had been separated by isoelectric focusing were identified by electrophoresis in acid-urea gels. As expected, the SV core was identified as a protein having a pI greater than 9.0. Polypeptides which focused in the pH range 6.1-6.7 were found to represent distinct charge isomers of E₁. Likewise, the polypeptides which focused between pH 8.0 and 8.6 were found to comprise charge isomers of E₂.

Some polypeptides which focused in the range of pH 8.0 to 8.6 migrated more rapidly than predicted in the second dimension creating two distinct zones just below the primary E₂ band. The faster migrating material may be due to interaction between E₂ and ampholytes during IEF. The same E₂ banding pattern was observed following extensive washing of IEF gels to remove ampholytes (data not shown). Another possible source of the artifact is the acid hydrolysis of E₂ during acid-end loading and isoelectric focusing, however, this possibility is considered unlikely since similar pH conditions are used for acid-urea gel electrophoresis in which E₂ migrates as a single zone.

The charge heterogeneity observed for SV E₁ was found to be independent of whether the sample was loaded at the acid or base end. When a shallow pH gradient (pH 4.0 to 8.0), which precluded the focusing of E₂ and core, was used to maximize resolution of E₁ isomers and the sample loaded at the base end, five charge isomers of E₁ were again detected. The pI values of these E₁ isomers were the same as those found following acid end loading of the samples.

SFV polypeptides which presented 4-5 peaks in isoelectric focusing gels were also identified by a second dimension electrophoresis in acid-urea gels. Three charge isomers were found for E₁ and three for E₂. The most acidic charge isomer of E₂ has the same pI as the most basic species of E₁. Thus, unlike E₁ and E₂ of SV, pI's of SFV E₁ and E₂ overlap making second dimension analysis necessary for identification of the individual charge isomers.

Effect of neuraminidase on the charge heterogeneity of SV and SFV polypeptides. The E₁ and E₂ glycoproteins of both AV and AFV are known to contain sialic acid residues (1,5,7). To determine if the charge isomers found for E₁ and E₂ of both viruses was due to a difference in the degree of sialylation, purified intact virions were treated with neuraminidase prior to isoelectric focusing. The pattern SV E₁ and E₂ charge isomers shifted to the more basic species. The shift was independent of either acid-end or base-end loading of the IEF gel. When neuraminidase treated SV was base-end loaded a conversion of E₁ charge isomers to the most basic species was even more clearly shown.

SFV exposed to neuraminidase under the same conditions used for SV and resulted in a similar shift of E₁ and E₂ charge isomers to the most basic species. When compared with the untreated SFV, only two charge components are seen and the majority of the charge species focus as the most basic polypeptide.

In the above study we used isoelectric focusing and polyacrylamide gel electrophoresis to examine the charge properties of polypeptides of SV and SFV grown in BHK cells. Our results show the glycoproteins of these two viruses significantly differ with respect to charge. In addition, each of the glycoproteins exists as charge isomers. Envelope proteins of both SV and SFV form multiple discrete bands upon isoelectric focusing in polyacrylamide gels. The heterogeneity observed was found to be independent of prior exposure to acid or alkaline pH and is independent of acid or base-end loading of IEF gels.

The SV envelope glycoproteins were each separated into two groups of five charge isomers ranging from pI 6.0 to 6.8 and pI 8.0 to 8.6. The separated groups of charge isomers were identified as E₁ and E₂ respectively by second dimension analysis in acid-urea gels. The SV core protein was found to have a pI greater than 9.0. Although the SV core protein has previously been reported to focus at pH 3.5 (2), the authors suggested that the apparent acid pI may have been due to the incomplete dissociation of viral RNA and the core protein. Consistent with this we have observed that SV core protein is not readily dissociable under the conditions reported by Dalrymple et al. (2).

Unlike SV, the envelope polypeptides of SFV did not separate into two groups of distinct bands upon isoelectric focusing. A single group of 4 charge isomers ranging from pI 6.7 to 7.5 was shown, by second dimension acid-urea gel electrophoresis to contain both E₁ and E₂. Three charge isomers were found for both E₁ and E₂. An overlap in pI also noted. The species of both E₁ and E₂ with the greatest negative charge were the least abundant. A comparison of the electrophoretic mobilities of the SV and SFV polypeptides in acid-urea gels pointed up a significant difference in the charge properties of the E₁ glycoproteins and core proteins. In addition, SFV glycoproteins E₁ and E₂ were reversed in their order of migration compared with that observed in SDS gels. Thus E₂, although of higher molecular weight than E₁, possesses a greater net positive charge at acid pH and migrates more rapidly than E₁ in the 5% gel.

It was found that the charge isomers of E₁ and E₂ for both SV and SFV are sensitive to neuraminidase treatment indicating a difference in sialic acid content for each isomer. Following treatment with neuraminidase, there was a consistent reduction in the acid forms of the charge isomers and a concomitant increase in the most basic isomer. Our data indicates that there is a neuraminidase resistant or unsialylated form present for each envelope glycoprotein. It should be noted that even after neuraminidase treatment, E₁ and E₂ of SV and SFV still have markedly different pI values. Table I summarizes the pI values of SV and SFV polypeptides with and without neuraminidase treatment.

Recently, analysis of genomic RNA from several alphaviruses indicated only a low degree of homology between SV and SFV (17). Our findings support this conclusion suggesting there may exist only a low degree primary structure similarity between proteins of the two viruses.

The finding of four neuraminidase-sensitive charge isomers for both E₁ and E₂ of SV indicates there are four potential sialylation sites on each molecule. If for SV the maximum number of sialic acid residues for

a type A oligosaccharide is two, then E₁ and E₂ may have two type A chains per molecule. Studies on the chemical composition of SV glycopeptides indicated the presence of only two oligosaccharide side chains per glycoprotein (1,12). Whether a type A chain can be substituted for a type B chain or vice versa on these polypeptides is unknown. Our data suggest that such substitutions are possible. Since SV glycopeptides are known to have a population of incompletely sialylated type A oligosaccharides (1), the unsialylated glycoproteins may possess two incomplete A chains or two complete A chains but lacking terminal sialylation or two B chains at the exclusion of an A chain. The finding of three charge isomers of SFV glycoproteins, two of which are neuraminidase sensitive, favors two possible sialylation sites per molecule of E₁ and E₂. If we assume that SFV has both A and B oligosaccharide side chains similar to SV, we predict a maximum of one A chain per molecule. The inability to add a second A chain may reflect a difficulty in substituting an A chain at a B chain attachment site. Again, the presence of unsialylated molecules of SFV as also found for SV may reflect the presence of an unsialylated A chain and not necessarily the absence of A chains on these molecules.

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Table 1

Neuraminidase Treated

V. Protein Organization in Flaviviruses

Electron microscopy of negatively stained preparations of flavivirus RHA show the presence of spike-like projections on the surface of the virions. These projections are most likely molecular arrays of envelope glycoprotein(s). The arrangement of the proteins forming such arrays is not known. The basic structural subunits of the alphaviruses, Sindbis virus and Semliki Forest virus appear to be heterodimers of the two envelope glycoproteins. These basic subunits may be organized as trimers to yield surface projections seen in the electron microscope, although this has yet to be demonstrated.

The organization of polypeptides on and in flavivirus particles can be determined using crosslinking methods similar to those used for alphavirus studies. In addition, once the organization of virion polypeptides is established, the crosslinking of the surface of infected cells will yield important information concerning the organization, morphogenesis, and immunology of flavivirus antigens. In this section we describe results of studies on the molecular organization of JEV.

When JEV RHA was exposed to 0.3 mM DMS a crosslinked (dimethyl-suberimide) product was generated whose molecular weight was double that of glycoprotein V3 (1978 Annual Report, Section VI). We extended these studies by examining the effects of increasing the concentration of crosslinking reagent in the reaction mixture. DTBP (dithiobispropionimide) at final concentrations of 0.5, 1 and 5 mM were used. Polypeptides V3 and NV2 appeared on the gels; however V2 and V1 had migrated off the gel. Exposure to 0.5 mM DTBP resulted in the formation of a single crosslinked 100,000 molecular weight product. Increasing the concentration of DTBP to 1 mM lead to the generation of two crosslinked products. A concentration of DTBP of 5 mM resulted in the formation of a third band. Molecular weight estimates of bands 2 and 3 could not be determined due to their close proximity to the top of the gel. Nevertheless, increasing the concentration of crosslinking reagent was shown to increase the number of crosslinked products, a predicted result.

A comparison of the noncleavable crosslinking reagent, DMS, and the reductively cleavable crosslinking reagent, DTBP, produced surprising results. Although both agents were equally effective in generating crosslinked products, the diversity formed were more numerous than was expected on the basis of the previous studies. Polypeptides were electrophoresed through 7.5% polyacrylamide gels, a change from conditions used previously; however, this could not account for the many new bands. A more likely possibility was the use in the latter experiments of newly synthesized crosslinking reagents. A test with the older reagents resulted in the formation of only three bands corresponding to the three highest molecular weight crosslinked products. The crosslinked products (XLP) have been assigned numbers corresponding to their estimated molecular weights.

Polypeptides from JEV infected cell lysates were readily detectable after electrophoresis in 12% polyacrylamide gels. Infected cells were exposed to Actinomycin D and cyclohexamide to reduce incorporation of label into host proteins. Uninfected BHK cells were labeled in the

absence of inhibitors but were otherwise treated as infected cells. Electrophoresis of control cell lysates showed the presence of numerous polypeptides, however only three of which comigrate with viral polypeptides. The three viral polypeptides are NV5, NV4 and NV3. Although these viral polypeptides could be contaminated with comigrating cell components this is considered unlikely since none of the non-comigrating polypeptides from the BHK cell lysate found in lysate of inhibitor-treated JEV-infected cell lysate.

These data indicate that inhibitor treatment was highly effective in blocking synthesis of cell polypeptides. The results of this initial study indicate that our technology is adequate for studies of the molecular organization of polypeptides on and in infected cells.

The crosslinking pattern of JEV RHA polypeptides becomes increasingly complex as the concentration of crosslinking reagent increases. The dramatic increase in complexity of the pattern when newly synthesized reagent is used points to the need for evaluating crosslinking reagent potency. Several enzymes are currently tested to determine if loss in enzyme activity can be correlated with the reactivity of the crosslinking reagent.

Based on the molecular weight of the crosslinked products, the order of appearance of crosslinked products and the known molecular weights of JEV RHA polypeptides the probable monomer composition of the products can be predicted. It should be noted that, with the exception of NV2, JEV RHA polypeptides migrate with the same mobility in the presence or absence of reducing agent; NV2 mobility is reduced from an estimated 25,000 to 22,000 in the absence of reducing agent (JEV data not shown but results were similar to DV RHA data. Since XLP100 has a molecular weight approximately twice that of V3, XLP100 is probably a homodimer of V3.

Similar analyses can be made for the other crosslinked products. For XLP125, a heterodimer composed of a V3 dimer and an NV2 monomer seems probable. Since XLP130 migrates as a relatively sharp band it probably does not contain NV2 but may be a trimer of V3 which migrates anomalously due to crosslinking induced configurational changes. Although the analysis could be continued for the other crosslinked products, the composition would only be suggestive. Analysis involving electrophoresis in the second dimension under reducing condition is required to unequivocally demonstrate the composition of these products.

Chronological List of All Publications Supported by the Contract

1. Cardiff, R. D. and J. K. Lund. 1976. The distribution of Denge-2 antigen by electron immunocytochemistry. Infect. Immun. 3: 1699.
2. Manning, J. S. and J. K. Collins. 1979. Effects of cell culture and laboratory conditions on type 2 Dengue. J. Clin. Micro. 10: 235.

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